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Multicopper Oxidases Enzymes use and its Characteristics

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Keywords*— *Multicopper oxidases. Microorganisms, Blue copper proteins, laccases.

***Abstract*—** *Multicopper oxidases are a large family of blue copper proteins which contain from one to six copper atoms per molecule. Their catalytic centre consists of three domains which involve type-1 Cu, type-2 Cu and a pair of type-3 Cu's. They include laccases, ferroxidases, ascorbate oxidase, bilirubin oxidase, laccase like multicopper oxidases. MCOs are capable of one electron oxidizing of aromatic as well as non-aromatic compounds with a concomitant four-electron reduction of molecular oxygen to water. These properties make them a valuable tool in various industries (food, textile, pharmaceutical) medicine or environment protection.*

I. INTRODUCTION

Multicopper Oxidases (benzenediol: oxygen oxidoreductase EC 1.10.3.2) are a family of enzymes that catalyse oxidation reactions of a substrate with simultaneous reduction of molecular oxygen to water. The universal catalytic centre is responsible for these reactions for all MCOs, composed of 4 or more copper atoms which form T1, T2 types and diatomic T3 types. Although the structure of the catalytic centre of most enzymes classified as MCO is similar, their biological functions and catalytic properties may be completely different. Various enzymes characterized by the presence of a catalytic centre typical of MCO and the ability to oxidize organic and inorganic compounds have been recognized. Many researchers have sought to analyse the characteristics on the basis of which it is possible to properly classify a newly identified enzyme which displays MCO properties. However, this problem still seems to be unresolved for most MCOs due to the highly similar properties of most substrates and considerable homology of amino acid sequences especially within the catalytic centre. The most commonly described MCOs include laccases especially from fungi belonging to the *Basidiomycota* division), and so called Laccase-like Multicopper Oxidases (LMCO), ascorbate oxidase (mainly described in higher plants), bilirubin oxidase (e.g. *Myrothecium verrucaria*), some fungal pigments and ferroxidases (*Saccharomyces cerevisiae* yeasts) [1].

II. MULTICOPPER OXIDASES: CLASSIFICATION, STRUCTURE AND PROPERTIES

A common feature of all enzymes included in the MCO family is the presence of a catalytic centre composed of at least four copper atoms divided on account of their spectroscopic and magnetic properties into three types: T1 and T2 types containing one copper atom and the diatomic T3 type [2]. Type T1 gives the enzyme molecule a blue colouring and exhibits intense light absorption at a wavelength of 610 nm, resulting from a covalent copper-cysteine bond. In turn the T2 type is colourless and, similarly to the T1 type, detectable by means of electroparamagnetic resonance spectroscopy (EPR). The T3 type does not exhibit activity in EPR spectroscopy as a result of antiferromagnetic coupling of copper atoms. However, it is distinguished by a light absorption band at a wavelength of 330 nm [3] (Fig. 1).

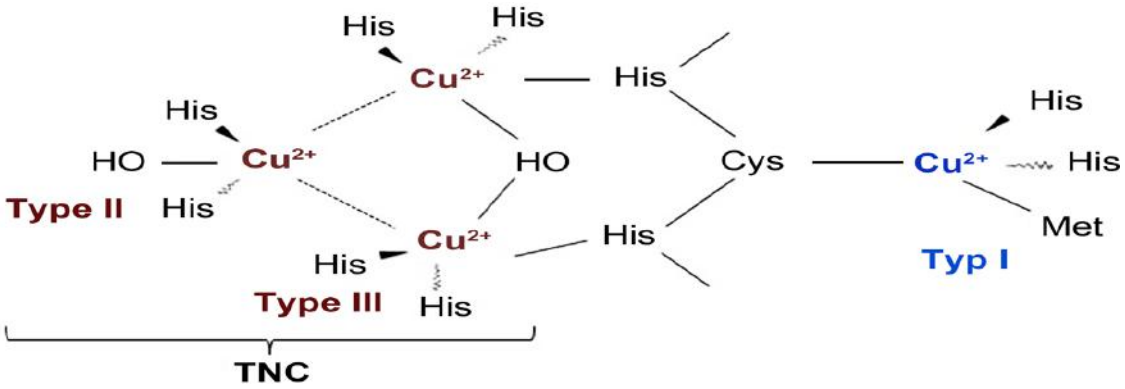


Fig: 1- Model of the catalytic cluster of MCO

In the T1 centre, where the substrate undergoes oxidation, the copper atom is bound to two histidine residues and one cysteine forming a distorted trigonal pyramid structure. The His-Cys-His sequence mentioned, which is characteristic of MCO, links T1 with T3. Sometimes the fourth amino acid residue, with weaker binding (most commonly methionine, leucine or phe-nylalanine), may occur in an axial position, which affects the oxidoreduction potential of the enzyme, stabilizes it and regulates its activity. The copper atom of T2 type and two atoms of T3 type, located in close proximity, are coordinated by the so called inter domain copper binding sites, composed of 2 and 6 histidine residues respectively, and forming a triatomic copper cluster. It is a structure unique for the MCO family and is the place where binding and the four electron reduction of molecular oxygen into water occurs [4]. The majority of MCOs contains about 500 amino acid residues and adopts the β-sheet layout in its secondary structure, shaped into the characteristic motif of the Greek Key {5]. Typically, an MCO molecule consists of three domains formed in this manner. The T1 copper centre is located in domain 3 (blue copper-binding domain), and the T2/T3 triatomic copper cluster is located at the interface between domains 1 and 3, which is farther away from the protein surface compared to domain 3.

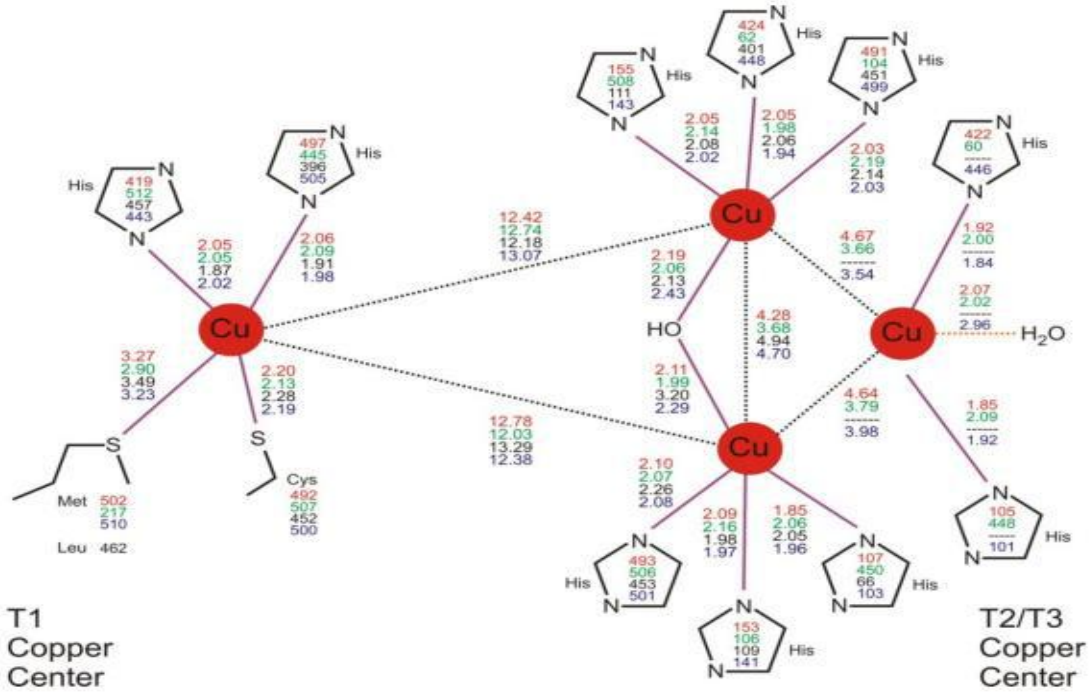


Fig. 2 : Overall arrangement of the reactive copper cluster in several representatives of the multicopper oxidase family. Residue number and distances between atoms are represented in colors: red, CotA laccase from Bacillus subtilis (PDB code: 1GSK); green, ascorbate oxidase from Cucurbita pepo (PDB code: 1AOZ); black, laccase from C. cinereus (PDB code: 1A65) and blue, CueO protein from E. coli (PDB code: 1KV7).

However, apart from the three-domain MCOs, proteins possessing two or six domains have also been characterized [6]. The MCO catalytic mechanism includes (1) the reduction of the T1 Cu site by capturing an electron from the oxidized substrate, (2) transferring the electron from the T1 site to the TNC and (3) reduction of O_2 with formation of two water molecules (Fig. 3).

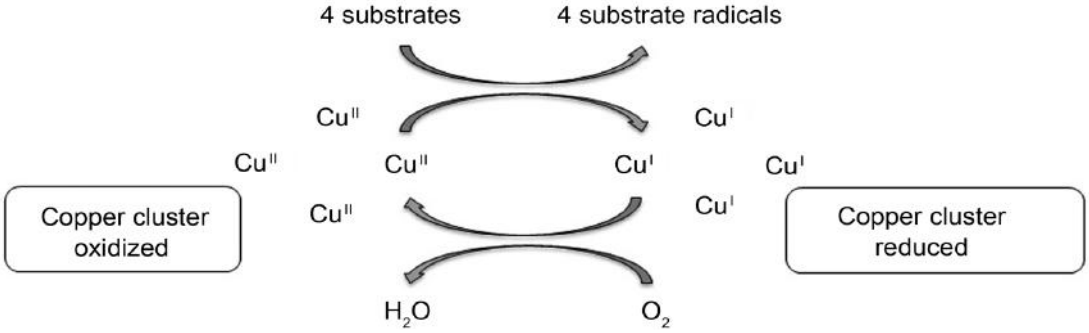


Fig: 3. Schematic of the catalytic mechanism of laccase.

MCOs oxidize a wide spectrum of substrates, such as phenol, methoxyphenol, aromatic amines, multi-aromatic compounds, metal ions [7]. MCO-catalysed reactions may occur directly (reactions of simple phenolic compound oxidation) or in the presence of a compound called a mediator, which mediates the transfer of electrons from the substrate to the active enzyme centre (Fig. 4).

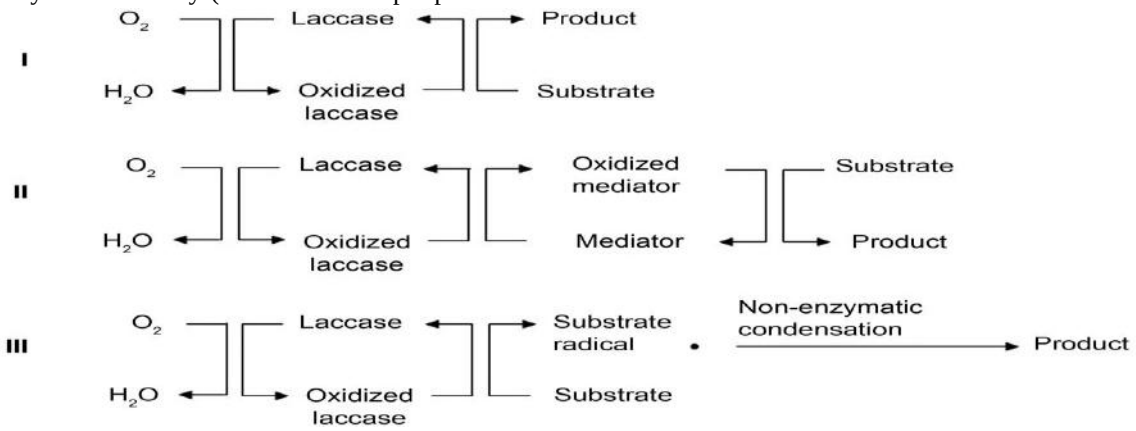


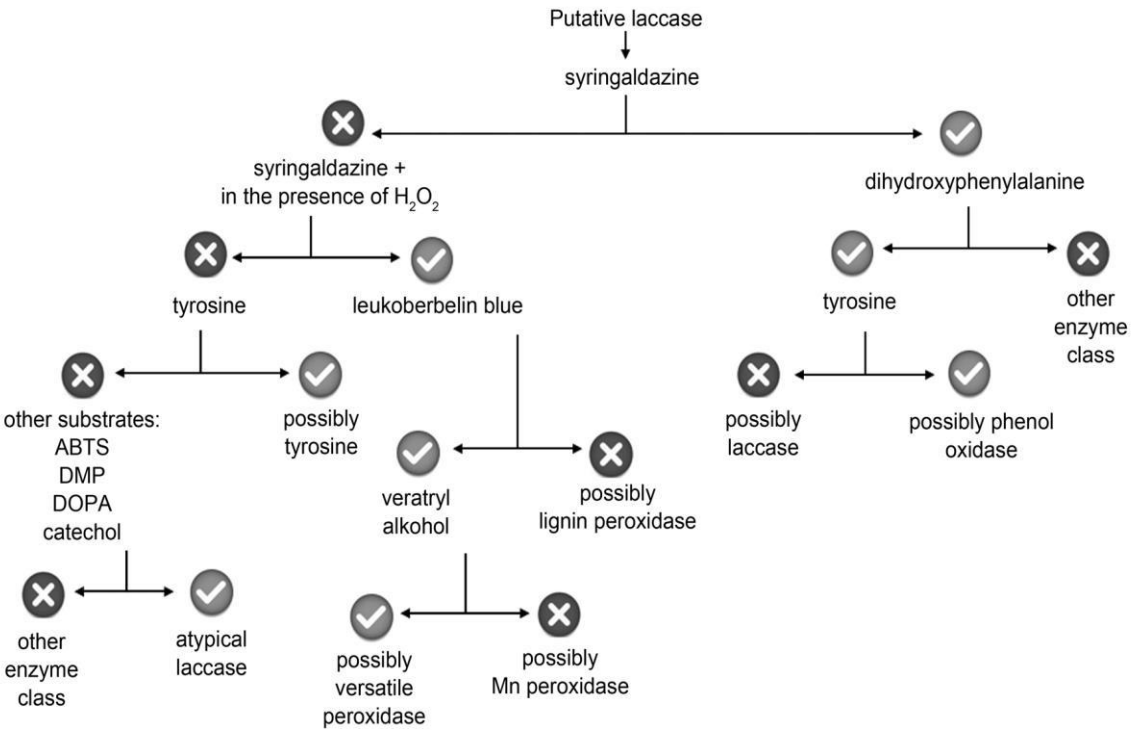
Fig 4. Mechanisms of reactions catalyzed by MCO.

If direct oxidation of the phenolic substrate leads to the formation of its reactive and unstable radicals, these may, in the process of non enzymatic, spontaneous coupling reactions combine to form dimers, oligomers or polymers [8]. MCOs are a very diverse group of enzymes produced by both prokaryotic organisms and *Eukaryota*, and are characterized by various, not yet fully understood, biological functions. Although all MCOs exhibit the capability for oxidizing aromatic compounds, two functional classes can be distinguished among them [86]. The first one is the enzymes that oxidize organic substrates more readily than metal ions. The group consists mainly of laccases and laccase-like enzymes. The latter, in turn, oxidize metal ions, such as Fe (II), Cu (I) and/or Mn (II), with higher efficiency, compared to organic substrates. The latter enzymes are referred to as metal oxidases, and the most common ones among them are human ceruloplasmin (Cp) and yeast ferroxidase [9]. The MCO division is not permanent and systematic, as there are no clear criteria for classification. For example, according to Hoegger *et al.* [10] multicopper oxidases form 10 enzyme groups: *Basidiomycota* laccases, *Ascomycota* laccases, insect laccases, MCO fungal pigments, fungal ferroxidases, plant and fungal ascorbate oxidases, plant-like enzymes like laccase, cooper resistance proteins (CopA), bilirubin oxidases and copper efflux proteins. In turn, Sirim *et al.* [11] distinguished within the MCO family: laccases, ferroxidases, ascorbate oxidases (AO) and bilirubin oxidases (BOD).

III. IDENTIFICATION METHODS OF MCOS

Until recently, the identification of enzymes included in the MCO was based mainly on the characteri-

stics of their biochemical features and catalytic abilities. MCO interactions with substrates can be broadly divided into two categories and one can distinguish enzymes with low substrate specificity and enzymes with high specificity. The plant and fungal laccases belong to the first category and they can oxidize diphenols, aryl amines and aminophenols, and their *Km* values are generally within the range of 1-10 mM. The remaining MCOs have a significant degree of substrate specificity (Km <1 mM) [12]. Some substances such as guaiacol, diammonium salt of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP) and catechol have long been termed model laccase substrates [13]. However, it turned out that many of them are also oxidized by other enzymes from the MCO group, e.g. LMCO or bilirubin oxidases. Syringaldazine used to be considered to be a specific laccase substrate [14]. Syringaldazine and ABTS can be converted by MnP or LiP. However, the oxidation which depends on H_2O_2 allows for distinguishing these enzymes from MCO. Multi Copper Oxidase can also be distinguished from MnP using the leucoberbelin blue dye test. This compound reacts specifically with manganese ions released by MnP, resulting in the emergence of blue colour exhibiting the maximum absorption of light at the wavelength of 620 nm [15]. Figure-4 presents a scheme for the identification and differentiation of laccases from other ligninolitic enzymes proposed by Fernandes *et al.* While the ability to oxidize specific substrates allows one to quickly determine the activity of a given enzyme from the MCO group, in the era of the development of highly advanced molecular techniques, it should not be the only method of identification and characterization of the newly recognized protein.



Fig; 5. The proposed scheme for the differentiation of laccases from other ligninolitic enzymes.

The development of omics techniques, such as genomics, transcriptomics and proteomics has contributed to determining the genes responsible for encoding enzymes, studying their expression at the level of the transcriptome and quantitative and qualitative analysis of the MCO against the background of other proteins in the body. Perry *et al.* [16] was the first to identify two genes encoding laccases *Agaricus bisporus* and found that these enzymes may exist as isoforms and be encoded by multigene gene families. Hence, hundreds of genes encoding MCO have been identified in both fungi and bacteria. The presence of many genes encoding enzyme isoforms in one strain may indicate the involvement of these enzymes in various physiological processes. The confirmation of this hypothesis may be the fact that individual enzyme isoforms often differ in substrate specificity and their activity may be different in different pH or temperature variants. Some of them have a constitutive character, and some may be induced, for example, by aromatic compounds or copper ions. Genomic and proteomic techniques have made it possible to identify consensus sequences for MCOs which distinguish them

from other enzymes. These sequences contain four (L1, L2, L3, L4) contiguous fragments of copper-binding amino acid residues, whose degree of similarity, depending on the organism from which the enzyme is derived, can range from 75 to 85%. The L2 and L4 regions allow the enzyme to be classified as MCO, whereas the L1 and L3 sequences indicate the subgroup of the enzyme [17].

IV. LACCASES VS. OTHERS MCOS

Among the microbial MCOs, laccases constitute the most studied and described group, being also the most numerous one. These are three domain MCOs which were isolated for the first time by *H. Yoshid in 1883* from resin from the *Rhus vernicifera* tree. Plant laccases, owing to the dehydrogenation mechanisms, play an important role in the polymerization of lignin-forming phenolic compounds, regeneration of damaged tissues and iron oxidation by converting Fe (II) to Fe (III) [15]. Although according to some researchers, the term laccase should be reserved exclusively for enzymes obtained from plants, also other three domain MCOs, e.g. of microbial origin, are called laccase if only they exhibit the ability to oxidize aromatic compounds [18].

Laccases of fungal origin most often occur in the form of several monomers which oligomerize and then form multimeric complexes. The average molecular weight of the monomer ranges from about 50 to 110 kDa. An important feature of fungal laccases is a carbohydrate group with covalent binding, usually constituting 10 to 45% of the total enzyme molecule and consisting mainly of mannose, N-acetylglucosamine and galactose. All these features protect fungal laccases against proteolysis, high temperatures, extremely high or low pH values and other unfavourable factors [74]. Among the fungi capable of the biosynthesis of laccases, the most numerous groups are those from the *Basidiomycota* division, e.g. *Lentinus tigrinus*, *Agaricus bisporus*, *Trametes versicolor*. Amongst them, there occur quite often the so-called fungi of white wood rot, incl. strains of the species *Phlebia radiate, Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Cerrena unicolor*. The synthesis of these enzymes has also been described in the fungi belonging to the *Ascomycota* division, e.g. *Aspergillus niger, A. oryzae*, *Neurospora* sp., *Trichoderma atroviride* and *T. harzianum* [19]. In fungi, laccases are involved in the processes of morphogenesis, lignin degradation and defence reactions to stress. These enzymes produced by saprophytic and mycorrhizal fungi are involved in the circulation of organic matter in the soil by degrading plant litter polymers or the formation of humic compounds [20]. Bacterial laccases have been identified in the cultures of strains including *Azospirillum lipoferum*, *Escherichia coli*, *Bacillus subtilis* and several species of *Streptomycetes* . They were also described in *Anabaena azollae* cyanobacteria. Bacterial laccases are characterized by greater activity and stability than fungal enzymes at high temperatures, at alkaline pH and in the presence of high concentrations of chlorine and copper ions [15]. Both phenol and non-phenol substrates can under golaccase mediated catalytic reactions. In the case of molecules with high redox potential and with large size particles, which are not able to independently penetrate into the active enzyme centre, the action of the so called mediators is indispensable. They are organic compounds of low molecular weight which, when oxidized by laccase, form highly active cationic radicals capable of oxidising non-phenolic compounds. The most commonly used synthetic mediators are ABTS, hydroxyanthranilic acid (HAA), hydroxybenzotriazole (HBT) and hydroxy-phthalimide (NPI). Natural mediators such as vanillin and syringaldehyde also have a similar effect [21].

Laccase-like multicopper oxidases (LMCO), similar to laccases, catalyse the oxidation of various substrates combined with the reduction of the O_2 molecule to two molecules of H_2O. Their biological functions are similar to the role of laccases, but not all of them have yet been recognized. LMCO have been described in many bacteria and fungi. The average molecular weight of LMCO is in the range of 51-66 kDa, while the number of amino acid sequences of enzymes is 470-600. LMCO of *Streptomyces* bacterium have also been described, whose molecular mass is 32.6 kDa, and the amino acid sequence length is 297. The optimum pH value for LMCO activity is not the same because it depends on the substrate used for its measurement [22]. For example, three types of LMCO originating from *Aspergillus niger*: McoA, McoB and McoG were purified and characterized for their bio-catalytic potential. All three enzymes were monomers with molecular weights in the range of 80 to 110 kDa. The highest McoA activity was observed in the pH 5.0 environments, while pH 6.0 was optimal for McoB and McoG. Additionally, McoA and McoB oxidized DMPPDA (N, N-dimethyl-p-phenylenediamine) in a wider pH range than McoG [89]. The LMCO isolated from *Myrothecium roridum* showed activity of both MCO and bilirubin oxidase .

Bilirubin oxidase is a thermostable enzyme conta-ining a disulphide bond. BOD catalyses the oxidation of tetrapyrroles, e.g. bilirubin to biliverdin as well as diphenols or aryl diamines with simultaneous reduction of four oxygen atoms to water [23]. This enzyme was discovered in the non-ligninolytic fungus *Myrothecium verrucaria* MT-1 in 1981 by Noriaki Tanaka and Sawao Murao. Unlike laccases, BODs are characterized by higher

activity and stability at neutral pH and high temperature, however not higher than 60°C. They are also characterised by high tolerance to chloride anions and other chelators. For example, bilirubin oxidase isolated from *Myrothecium verrucaria* (MvBOD) exhibited the highest catalytic activity in the temperature range of 30-60°C and pH from 7 to 8.5. However, in solutions with pH above 9, a decrease in BOD catalytic activity by as much as 50% has been observed [24].

Another example of MCO is SLAC, a two-domain multicopper oxidase described in *Streptomyces coelicolor*, which exhibits the ability to oxidize aromatic and non-aromatic compounds containing amino and hydroxyl groups. Due to the similarity of the sequence to fungal laccases, but also smaller size, this enzyme has been defined as a small laccase. On the basis of the comparative analysis of protein sequences, the similarity of SLAC and other laccases in the position of metal ligands has been established. However, the occurrence of 24 histidine residues in the SLAC sequence has been established, which may indicate its role in binding the excess of intracellular copper ions in order to transfer them during export through the TAT secretory system [25]. SLAC is characterized by resistance to reducing compounds and thermal stability. In addition, this enzyme exhibits the highest activity in the environment with pH 9. Owing to these features, SLAC have found their application in the pulp and paper industry for dye decolorization.

V. APPLICATION OF MULTICOPPER OXIDASES

On account of the ability to oxidize many substrates, extracellular character and fairly high stability in a wide range of pH and temperatures, the MCO enzymes are characterized by a high application potential. So far enzymes from this group have found application in environmental protection, medicine, pharmaceutical industry, cosmetics and in the food industry. MCO enzymes have been applied in many areas of the food industry, like baking, vegetable and fruit processing, winemaking and brewing. The bakery industry commonly uses laccases to improve bread structure, as well as the flavour and durability of pastries [26]. However, it has been proven that other MCOs, such as bilirubin oxidase, can be used to cross-link biopolymers by improving the physicochemical properties of food products. The process increases the durability and stability of dough, at the same time reducing its viscosity. This effect was noted especially when using lower quality flour [27]. MCO is used, e.g., for cross-linking arabinoxylans so that the created network of transverse polymer bonds has a positive effect on crumb and crustiness of bread. Laccases can be used instead of physical adsorbents like SO_2 to eliminate undesirable phenol derivatives, causing darkening and clouding of fruit juices, beers and wines. New reports have appeared lately, indicating the possible use of MCOs isolated from cultures of lactic acid fermentation bacteria, among others for removing biogenic amines from wine and some oriental cuisine products.

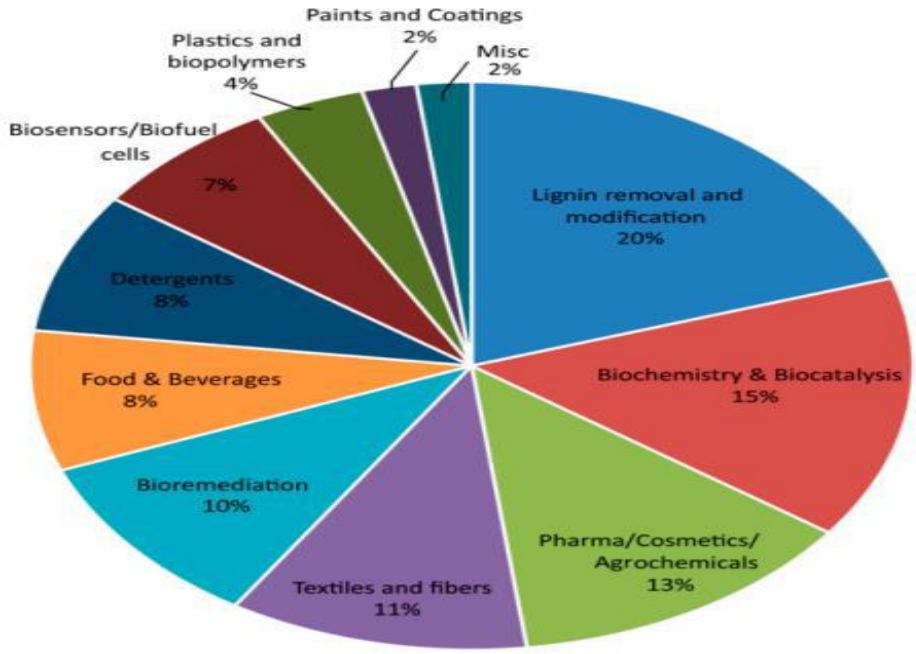


Fig.6: Applications of multicopper oxidases

The use of MCO in the food industry is quite common. Preparations available on the market, such as Falouvorstar, Suberase or LACCASE Y120, which are based on laccase activity, are successfully used in brewing, production of corks for the wine industry and improvement of the colour values of food products. Enzymes from the MCO group are used as biocatalysts in the reactions yielding many active substances which are components in the composition of household chemistry, body care products and medicines characterized by antimicrobial and antioxidant activity. Such activity is exhibited by molecular iodine (I2), whose preparation through oxidising it has been described for MCOs isolated from the culture of *Alphaproteobacterium* sp. Q-1 and *Roseovarius* sp. A-2. Strong antifungal activity has also been proven for iodinated phenolic compounds obtained in the reaction catalysed by laccase. Laccases are also successfully used as biocatalysts in the synthesis of drugs, among others β-lactam antibiotics and anti-cancer agents, e.g. vinblastines or mitomycin. Since 2006, when antiproliferative activity of laccase was demonstrated for the first time, intensive research has been conducted on the use of this enzyme as an anti-cancer agent [28]. The ability to inhibit cell division of breast, liver, colon and prostate cancer has been proven for laccases from various species of basidiomycetes. The application of laccase manufactured by *Cerena unicolor* in the treatment of blood and cervical cancer has been demonstrated and covered by patent protection. This enzyme, added in the right concentration, had a strong cytotoxic effect on cervical cancer cells of the SiHa and CaSki line and did not affect adversely the fibroblast cells constituting the reference system.

VI. SUMMARY

Multi Copper Oxidase proteins are enzymes containing from one to six atoms of copper per molecule. Multicopper oxidases include laccases, ferroxidases, ascorbate oxidase, bilirubin oxidase, some fungal pigments with multicopper oxidase character and the so-called laccase-like enzymes. MCOs possess the ability to oxidize both organic and inorganic compounds. The reactions catalysed by MCO are accompanied by the reduction of molecular oxygen to water. These properties make them a valuable tool in bioremediation processes, medicine, pharmaceutical industry, cosmetics and food industry.

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